

Dose-response analysis of short-term effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in three differentially susceptible rat lines

Ulla Simanainen,* Jouni T. Tuomisto, Jouko Tuomisto, and Matti Viluksela

National Public Health Institute, Department of Environmental Health, P.O. Box 95, FIN-70701 Kuopio, Finland

Received 2 July 2002; accepted 13 December 2002

Abstract

Line A, B, and C rats were selectively bred from TCDD-resistant Han/Wistar (Kuopio; H/W) and TCDD-sensitive Long-Evans (Turku/AB; L-E) rats. Line A rats are the most resistant to TCDD acute lethality followed by line B and line C rats. The resistance in line A rats is associated with a mutated H/W-type aryl hydrocarbon receptor (*Ahr*) allele (*Ahr^{hw}*) and in line B rats the resistance is associated with an allele of an unknown gene *B* (*B^{hw}*), while line C rats are almost as sensitive to TCDD as L-E rats. The dose-responses of characteristic short-term effects (day 8 postexposure) of TCDD were used to evaluate the efficacy (magnitude of effect) and potency relationships between these lines. Line A rats showed similar efficacies as line C (line A:line C efficacy ratio more than 0.7) for thymus weight, EROD activity, and incisor tooth defects. In contrast, efficacies in line A were decreased (efficacy ratios 0.19–0.37) for body weight change, serum bilirubin, and FFA levels, and serum ASAT activity. For most endpoints the efficacies in line B rats seem to be lower than in line C rats. The potencies were close to each other in line A and B rats, but somewhat lower than in line C rats. The results support our previous concept of two different AHR-mediated signaling pathways leading to dioxin type I and type II endpoints. Rats with the *Ahr^{hw/hw}* genotype show a markedly decreased efficacy for type II endpoints, but *B^{hw}* allele had only a minor effect on efficacies for most endpoints. Both H/W-type resistance alleles also decreased the potency of TCDD. However, the potency differences in short-term toxicity seem not to explain, at least alone, the differences seen in acute lethality among the rat lines.

© 2003 Elsevier Science (USA). All rights reserved.

Keywords: Dioxin; TCDD; Strain differences; AHR; Efficacy

Introduction

Exposure of experimental animals to the environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) causes a set of diverse toxicological and biological effects seen in most vertebrates studied. Studies with different mouse models (Birnbaum et al., 1990; Poland and Glover, 1975; Poland et al., 1976) have revealed that the TCDD toxicity is mediated by a soluble, intracellular protein, the aryl hydrocarbon receptor (AHR). In addition, the lack of TCDD-induced toxicity and transcriptional activation of genes encoding xenobiotic metabolizing enzymes in AHR-knockout mice (Fernandez-Salguero et al., 1996) strongly support the hypothesis that many biochemical and toxic effects of TCDD and related compounds are AHR-dependent.

In TCDD responsive C57BL/6J and nonresponsive DBA/2 mouse strains, the strain difference in ligand-binding affinity of the AHR causes a 10- to 14-fold difference in the potency of TCDD for acute lethality (Chapman and Schiller, 1985; Ema et al., 1994; Okey et al., 1989; Poland et al., 1994). These mouse strains also showed similar potency differences in sensitivity to nonlethal endpoints of TCDD toxicity, including CYP1A1 induction. However, TCDD efficacy (magnitude of effect) was only slightly affected. Similarly, the potency difference in acute lethality between congenic C57B1/6J mice was also seen for body weight and organ weight changes as well as clinical pathology effects, while efficacy was only slightly different (Birnbaum et al., 1990).

In our laboratory we have used a rat model based on an exceptionally wide (>1000-fold) sensitivity difference between the sensitive Long-Evans (Turku AB) (L-E) rats and the resistant Han/Wistar (Kuopio) (H/W) in terms of acute

* Corresponding author. Fax: +358-17-201-265.

E-mail address: ulla.simanainen@ktl.fi (U. Simanainen).

lethality of TCDD. A point mutation in the H/W-type *Ahr* allele (*Ahr*^{hw}; hw denoting allele originally from H/W rats) results in an abnormal C-terminus transactivation domain and a smaller AHR protein compared to normal rat strains (*Ahr*^{wt}; wt denoting wild-type allele) (Pohjanvirta et al., 1998, 1999). The exceptional resistance of H/W rats to TCDD acute lethality is associated with this abnormal AHR and, to a lesser extent, with an unknown gene “B” (Tuomisto et al., 1999).

The dose-responses for typical endpoints of dioxin toxicity were previously modeled and differences quantified in L-E and H/W rats (Simanainen et al., 2002). Despite the large difference in LD50 values, the potency of TCDD for nonlethal biochemical and toxic short-term effects was much less affected by the mutated H/W-type AHR. Furthermore, the endpoints could be divided into two different categories, type I and II endpoints, by using the efficacy difference between the strains as the classification criterion (Simanainen et al., 2002; Tuomisto et al., 1999). Type I endpoints (EROD activity, thymus weight, tooth defect) showed similar efficacy in both strains, while for type II endpoints (body weight, serum FFA and bilirubin levels, serum ASAT activity) the efficacy was less than half in H/W rats compared to L-E rats.

We used conventional cross-breeding methods to segregate the H/W rat resistance genes, *Ahr* and *B*, into different rat lines each harboring the resistant alleles from only one of these two genes (Tuomisto et al., 1999). H/W and L-E were the parent strains used in the breeding, and the new rat lines were designated lines A, B, and C. Line A has the mutated *Ahr*^{hw} allele and the wild-type *B* allele (genotype *Ahr*^{hw/hw} *B*^{wt/wt}). Line B lacks the resistant *Ahr*^{hw}, but is homozygous for *B*^{hw} (*Ahr*^{wt/wt} *B*^{hw/hw}). Line C possesses neither of the resistance alleles (*Ahr*^{wt/wt} *B*^{wt/wt}). Lines A, B, and C exhibit highly different LD50 values for TCDD: >10,000, 830, and 40 µg/kg in males, respectively, and >2000, 410, and 19 in females, respectively. Thus, line A is as resistant as H/W, line C is almost as sensitive as L-E, and line B is intermediately resistant.

In this study we used these new rat lines to further characterize the roles of the resistance alleles *Ahr*^{hw} and *B*^{hw} in short-term effects of TCDD, especially with regard to the efficacy and potency differences as previously demonstrated in the parent strains. Furthermore, the results were expected to help to define the dependence of type I and II responses on the resistance alleles and to further understand the mechanism of TCDD toxicity.

Materials and methods

Chemicals

TCDD was purchased from the UFA-Oil Institute (Ufa, Russia) and was over 99% pure as confirmed by gas chromatography–mass spectrometry. It was weighed and dis-

solved in diethyl ether. Adjusted volume of diethyl ether was mixed with corn oil and ether was let evaporate. Dosing solutions were mixed in a magnetic stirrer and sonicated for 20 min before dosing. Methanol and diethyl ether were of analytical grade and purchased from Merck (Darmstadt, Germany) and from BDH Laboratory Supplies (Poole, UK), respectively. All other chemicals used in assays were of analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Animals and animal husbandry

Adult female line A, B, and C rats were obtained from the breeding colony of the National Public Health Institute (Kuopio, Finland). The crossing protocol to develop these lines has been described by Tuomisto et al. (1999). The breeding colony is kept in a specific pathogen-free (SPF) barrier unit and the animals are regularly subjected to a health survey consisting of serological, bacteriological, and parasitological screening as suggested by FELASA (1996). These surveys indicated that the animals were free of typical rodent pathogens.

The rats were 8–12 weeks old at the beginning of the experiment. Line A rats weighed 176 ± 20 g (mean \pm SD); line B rats weighed 198 ± 20 g, and line C rats weighed 178 ± 13 g. They were housed in stainless steel wire-bottom cages, five to six rats per cage. Rats received commercial rat chow (R36; Lactamin, Stockholm, Sweden) and tap water ad libitum. The ambient temperature in the animal room was $21 \pm 1^\circ\text{C}$, and the relative humidity was $50 \pm 10\%$. The rats were kept under a photoperiodic cycle of 12 h light/12 h dark in an air-conditioned animal room.

Experimental design

Rats were randomly divided into experimental groups of five to six animals and given a single oral dose of TCDD in corn oil by oral gavage using a metal cannula with a ball tip (Day 0). The dosing volume was 4 ml/kg. TCDD doses had ca. threefold intervals and were between 0.03 and 3000, 0.03 and 1000, and 0.03 and 100 µg/kg body weight in line A, B, and C rats, respectively. Control animals were dosed similarly with corn oil vehicle. On Day 8 postexposure, rats were decapitated with a guillotine, trunk blood was collected, and serum was separated. Liver and thymus were quickly removed, trimmed, and weighed. Liver samples were quickly frozen in liquid nitrogen. Serum and liver samples were stored at -80°C until analyses.

Biochemical analyses

Ethoxyresorufin-O-deethylase (EROD) activity. EROD activity in liver S9 fraction was assayed fluorometrically according to Kennedy and Jones (1994) with slight modifications. The assay was carried out using 96-well microtiter plates and a protein concentration of 30 µg/well. Samples

were incubated for 3 min at 37°C, and the linearity of EROD activity with time was confirmed. Protein concentration was measured by the Bradford (1976) method using the Bio-Rad protein assay reagent and bovine serum albumin standards (Bio-Rad, Hercules, CA, USA).

Serum ASAT activity, FFA concentration, and total bilirubin concentration. Serum aspartate aminotransferase (ASAT) activity assay was based on kinetic measurement using UV photometric detection, and it followed the Scandinavian Committee on Enzymes (S.C.E.) of the Scandinavian Society for Clinical Chemistry and Clinical Physiology recommendations (1974). Serum FFA concentrations were analyzed according to the method of Shimizu et al. (1980). Serum total bilirubin was measured using a modified diazo ultramicromethod of Walters and Gerarde (1970). All these assays were performed with the Kone Specific selective chemistry analyzer (Kone Instruments, Espoo, Finland).

Tooth examination

Lingual attrition surfaces of lower incisors were examined for the presence and severity of pulpal perforations (Alaluusua et al., 1993) using a stereomicroscope. Severity of pulpal perforation was scored semiquantitatively using a scale of 0–3 (0, no perforation; 1, initial perforation; 2, perforation; 3, perforation with pulpal hemorrhage). Teeth were examined by one investigator not knowing the dose.

Statistical analyses and curve fitting

Intrastrain dose group comparisons were carried out using the one-way analysis of variance (ANOVA). If this test showed a significant difference, the least significant difference (LSD) test was used as a post-hoc test. In case of nonhomogenous variances (according to Levene's test, $p < 0.01$), the nonparametric Kruskal–Wallis ANOVA was used, followed by the Mann–Whitney U test.

Dose-response curves were fitted assuming a shape of cumulative normal distribution after logarithmic dose transformation and using the least-squares method with sequential quadratic programming (SPSS 10.0 Statistical Program, SPSS Inc., Chicago, IL). The regression analysis was performed separately for all strains and endpoints using the formula,

$$E(d) = E_{\min} + (E_{\max} - E_{\min}) * \Phi((d - \mu)/\sigma),$$

where $E(d)$ is the observed effect at $d = \log(\text{dose})$, E_{\min} is the control effect, E_{\max} is the maximal effect, μ is $\log(\text{ED50})$, σ is $\log(\text{GSD})$ (GSD denoting for geometric standard deviation), and Φ is the cumulative standard normal distribution.

The following restrictions were used for input parameters: $\text{ED50} \geq 0.01 \mu\text{g/kg}$, $\text{ED50} \leq 3000 \mu\text{g/kg}$; $\text{GSD} > 1.02$; $\text{GSD} < 50$; $E_{\min} \geq 0$; $E_{\max} \geq 0$.

Parameter estimates were compared between strains. A probability that one estimate is smaller than the other was calculated by randomly sampling values from the likelihood distributions of the two estimates. The difference between strains was considered statistically significant if this probability was smaller than 0.025 or greater than 0.975.

To confirm the obtained results, the data were also modeled using nonlinear regression with a modified hyperbolic curve, the Hill equation (De Vito et al., 1997, Holford and Sheiner, 1981).

$$E(D) = E_{\min} + ((E_{\max} - E_{\min}) * D^n) / (\text{ED50}^n + D^n),$$

where $E(D)$ is the observed effect at $D = \text{dose}$, E_{\min} is the control effect, E_{\max} is the maximal effect, ED50 = effective dose 50%, and n = Hill coefficient, the shaping factor.

Efficacy (or relative efficacy) was defined as a relative change of the effect from the control level: $(E_{\max} - E_{\min}) / E_{\min}$. Absolute efficacy was defined as an absolute change: $E_{\max} - E_{\min}$. Efficacy ratio was defined as the relative efficacy in one line divided by that in the reference line (usually C). Sometimes absolute efficacy ratio was calculated by using absolute efficacies. Potency was defined as the inverse of ED50 .

Results

All endpoints measured were significantly and dose-dependently affected by TCDD in all lines, except serum FFA levels in line A rats (Figs. 1 and 2). ED50 , E_{\min} , E_{\max} , and GSD estimates for all endpoints are shown in Table 1. Liver EROD activity was a low-dose effect with ED50 below $1 \mu\text{g/kg}$, while serum FFA and bilirubin levels as well as ASAT activity were high-dose effects with ED50 above $20 \mu\text{g/kg}$. Incisor tooth defect as well as body weight change and thymus weights could be classified as intermediate effects. Body weight change and tooth defect were clearly closer to the high-dose effects (ED50 in line C rats $\geq 5.5 \mu\text{g/kg}$ and up to $35 \mu\text{g/kg}$ in line A rats). Thymus weight was closer to the low-dose effects (ED50 in line A, B, and C rats 0.83 – $1.9 \mu\text{g/kg}$).

Similar to our earlier findings (Simanainen et al., 2002), liver EROD activity showed decreases from maximal values at high-dose levels due to liver toxicity. For this reason data from the highest doses of >30 , >10 , and $>3 \mu\text{g/kg}$ TCDD for lines A, B, and C, respectively, were not used for the dose response modeling of CYP1A1 induction (measured as EROD induction).

Efficacy

The estimates for E_{\min} and E_{\max} seemed to be robust and had narrow confidence intervals for most endpoints measured. However, E_{\max} confidence intervals for serum bilirubin levels and ASAT activity were large in line A rats.

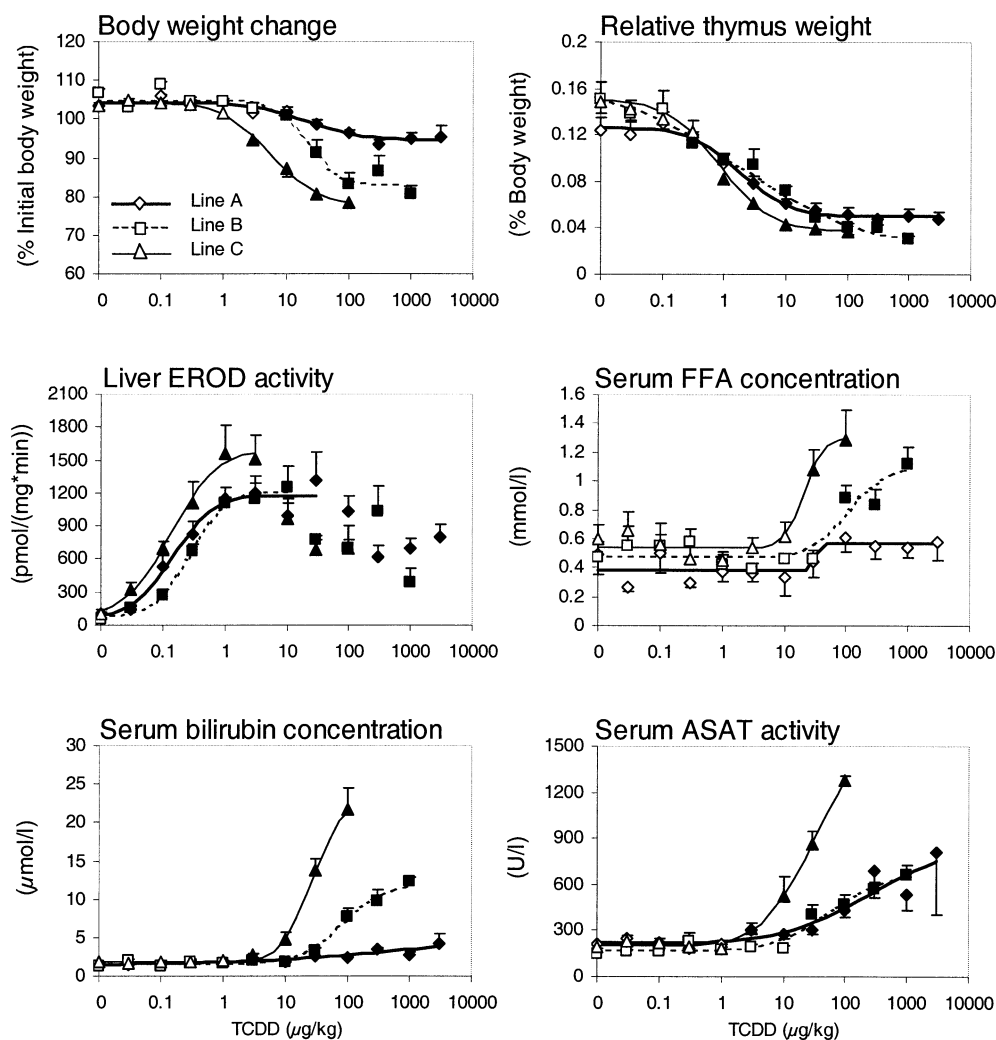


Fig. 1. Modeled dose-responses and group means (five to six rats per treatment group) \pm SE of relative body weight change, body-weight-related thymus weight, liver S9 ethoxyresorufin *O*-deethylase (EROD) activity, serum free fatty acid concentrations (FFA), serum aspartate aminotransferase (ASAT) activity, and serum total bilirubin concentration in line A, B, and C rats 8 days after exposure to a single oral dose of TCDD. Dose-response curves were fitted assuming a shape of cumulative normal distribution after logarithmic dose transformation and using the least-squares method with sequential quadratic programming. The curves are drawn up to the highest dose modeled. For statistically significant group means ($p < 0.05$) differences versus corresponding controls are depicted with solid symbols.

E_{\min} estimates were similar among the strains, except that line A tended to have larger thymus, smaller serum FFA levels, and more tooth defects. Some E_{\max} estimates were beyond the experimental data, representing an extrapolated part of the dose-response with large confidence intervals (serum ASAT activity and bilirubin concentration in line A, thymus weight in line B). These extrapolations, however, did not affect the interpretation of the results.

Efficacy estimates expressed as relative changes in the magnitude of response ($(E_{\max} - E_{\min})/E_{\min}$) and the efficacy ratios (compared to line C) are shown in Table 2. For most endpoints the efficacy was highest for line C rats and lowest for line A rats. Line A rats had significantly smaller efficacies than line C and line B rats for all endpoints previously defined as type II endpoints except ASAT (A–B difference for bilirubin was almost significant). This was

Mean severity of incisor tooth defects

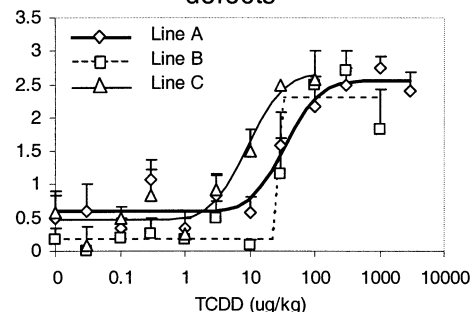


Fig. 2. Modeled dose-responses and group means (five to six rats per treatment group) \pm SE of mean severity of right and left incisor tooth defects in line A, B, and C rats 8 days after exposure to a single oral dose of TCDD. Dose-response curves were fitted assuming a shape of cumulative normal distribution after logarithmic dose transformation and using the least-squares method with sequential quadratic programming.

Table 1

Estimates (CI 95%) for E_{\min} , E_{\max} , ED50 ($\mu\text{g/kg}$), GSD, percentage of variance explained (R^2), and Hill coefficient (n) for different effects in line A, B, and C rats 8 days after a single oral exposure to different doses of TCDD

Rat line	Body weight change (% initial body weight)	Relative thymus weight (% body weight)	Liver EROD activity ((pmol/mg) \times min)	Serum FFA levels (nM)	Serum bilirubin levels (μM)	Serum ASAT activity (U/l)	Incisor tooth defects
E_{\min}							
A	104 (103–105)	0.13 (0.12–0.14)*	72 (0–390)	0.38 (0.30–0.45)* [†]	1.6 (1.1–2.1)	217 (102–330)	0.59 (0.33–0.85) [†]
B	105 (104–107)	0.16 (0.12–0.20)	85 (0–250)	0.48 (0.41–0.55)	1.7 (1.2–2.1)	175 (140–210)	0.20 (0–0.45)
C	104 (103–106)	0.15 (0.14–0.16)	88 (0–570)	0.54 (0.45–0.64)	1.9 (0.98–2.8)	203 (150–250)	0.46 (0.2–0.73)
E_{\max}							
A	95 (93–96)* [†]	0.050 (0.043–0.058)*	1170 (1000–1300)*	0.57 (0.47–0.66)* [†]	5.0 (0–14)* [†]	878 (0–2000)	2.6 (2.2–3.0)
B	83 (80–86)*	0.026 (0–0.055)	1220 (1100–1400)	1.1 (0.84–1.3)	12 (11–14)*	668 (540–800)*	2.3 (1.9–2.7)
C	78 (74–81)	0.038 (0.028–0.047)	1580 (1200–2000)	1.3 (1.0–1.5)	23 (18–28)	1620 (840–2400)	2.7 (2.0–3.3)
ED50 ^a							
A	20 (9.1–46)*	1.8 (0.94–3.3)	0.15 (0.064–0.34)	32 (0–3000)	656 (0.0017–3000)	283 (0.37–3000)	35 (17–75)*
B	21 (14–33)*	1.9 (0.44–8.5)	0.28 (0.17–0.47)	110 (39–310)*	95 (61–150)*	57 (25–130)	30 (0–3000)
C	5.5 (3.7–8.4)	0.83 (0.54–1.3)	0.14 (0.053–0.35)	21 (11–40)	27 (18–40)	33 (9.2–120)	9.3 (4.4–20)
GSD							
A	5.0 (1.7–15)	4.4 (1.9–10)	3.4 (1.1–11)	1.2 (0–50)	50 (0.012–50)	15 (0.063–50)	2.7 (0.96–7.3)
B	2.8 (1.5–5.1)	34 (2.5–50)	2.9 (1.4–6.0)	3.1 (0.83–12)	3.6 (2.1–6.2)	5.1 (1.8–14)	1.0 (0–50)
C	4.2 (2.5–7.2)	4.8 (2.6–8.7)	4.0 (0.92–17)	1.8 (0.90–3.6)	2.5 (1.5–4.2)	4.9 (1.8–13)	2.8 (1.0–7.5)
R^2							
A	0.69	0.77	0.65	0.13	0.42	0.31	0.57
B	0.80	0.79	0.81	0.51	0.88	0.76	0.59
C	0.91	0.90	0.70	0.48	0.88	0.90	0.61
Hill n^b							
A	1.1 (0.25–1.9)	1.1 (0.41–1.8)	1.3 (0–2.7)	5.0 (0–200)	0.31 (0–1.1)	0.75 (0–2.1)	1.8 (0–3.8)
B	1.5 (0.55–2.5)	0.42 (0.068–0.78)	1.5 (0.34–2.7)	5.0 (0–40)	1.3 (0.71–1.9)	1.0 (0.33–1.6)	5.0 (0–74)
C	1.1 (0.67–1.6)	1.0 (0.60–1.5)	1.1 (0–2.5)	2.8 (0–6.7)	1.8 (0.82–2.7)	0.94 (0.40–1.5)	1.6 (0–3.36)

^a $\mu\text{g/kg}$ TCDD.

^b Hill coefficients were derived from the Hill plot model for the same data.

* Significantly different from line C estimate.

[†] Significantly different from line B estimate.

also true when efficacy was measured as absolute changes ($E_{\max} - E_{\min}$; not shown). Efficacy ratios between line A and C rats were 0.19–0.44 for all type II endpoints. In contrast, they were 0.81, 0.90, and 0.70 for thymus weight,

liver EROD activity, and tooth defect, respectively (type I endpoints). The efficacies for thymus weight were significantly different between lines A and C. The efficacy ratios between line B and C were 0.57–0.93, except for serum

Table 2

Efficacy ($(E_{\max} - E_{\min})/E_{\min}$), and relative efficacy and potency (compared with line C) for different effects in line A, B, and C rats 8 days after a single oral exposure to different doses of TCDD

Rat line	Body weight change	Relative thymus weight	Liver EROD activity	Serum FFA levels	Serum bilirubin levels	Serum ASAT activity	Incisor tooth defects
Efficacy ^a $((E_{\max} - E_{\min})/E_{\min})$							
A	−0.093* [†]	−0.60*	15	0.5* [†]	2.2*	3.1	3.31
B	−0.21	−0.84	13	1.3	6.5	2.8	10.62
C	−0.25	−0.74	17	1.4	11	7.0	4.74
Efficacy relative to line C							
A	0.37	0.81	0.90	0.37	0.19	0.44	0.70
B	0.84	1.1	0.78	0.93	0.57	0.40	2.24
C	1	1	1	1	1	1	1
Potency relative to line C							
A	0.27	0.47	0.93	0.66	0.041	0.12	0.26
B	0.26	0.43	0.48	0.19	0.28	0.58	0.31
C	1	1	1	1	1	1	1

^a Statistically significant ($p < 0.05$) differences are shown only for efficacy.

* Significantly different from line C estimate.

[†] Significantly different from line B estimate.

ASAT activity (0.40) and dental defects (2.24). The high efficacy for tooth defects in line B rats was due to a relatively low E_{\min} estimate; absolute efficacies were similar in line B and C.

Potency

The potencies relative to line C were calculated using ED50 estimates (Table 2). The ED50 estimates were robust and had narrow confidence intervals for all other endpoints except for serum bilirubin levels, FFA levels, and ASAT activity in line A rats as well as tooth defects in line B rats. In the classical receptor theory, the calculation of potency ratios for receptor–ligand interactions are theoretically sound only for full agonists, i.e., for compounds producing the maximal response. Because in this study the E_{\max} estimates for certain endpoints were not the same in all rat lines, the comparison of potencies of these endpoints can only be considered as an indicative tool for potency differences.

TCDD was more potent in line C rats than in line A and B rats for all endpoints (Table 1). The ED50 values were 1.1–5.2 times higher in line A and B rats than in line C rats (excluding unreliable estimates for bilirubin level and ASAT activity in line A rats). However, the potency difference was statistically significant only for body weight change and tooth defect (line A vs line C), and for body weight change, serum bilirubin, and FFA (line B vs line C). ED50 estimates did not differ significantly between lines A and B.

Hill coefficient

The corresponding ED50 values derived from Hill plot were consistent with those derived from the log normal distribution model (not shown). The Hill coefficient (n ; the shaping factor) for each endpoint and rat line are presented in Table 1. A Hill coefficient greater than 1.0 indicates a positive and a coefficient less than 1.0 indicates a negative ligand cooperativity. When the cooperativity is positive, the increase in the dose at low dose levels causes only a minor response until the threshold dose where only a small increase in dose causes a dramatic increase in response. In negative cooperativity the dramatic increase in response occurs at low doses and then the response is much less sensitive to increase in dose and becomes almost independent of the concentration of ligand. For most endpoints the factor was close to 1. The exceptions were serum FFA level and line B tooth defects with factors between 2.8 and 5.0 as well as line B relative thymus weight (0.42) and line A serum bilirubin (0.31).

Discussion

Characteristic short-term effects of TCDD other than lethality were evaluated among line A, B, and C rats. The

initial observations on line A, B, and C rat sensitivity differences are documented in a previous article by Tuomisto et al. (1999). In this study we determined comprehensive dose-response curves for short-term effects of TCDD and carried out a quantitative comparison among the rat lines using modeled potency and efficacy estimates for each line and endpoint. Analysis of the outcome is complicated by the fact that the biochemical and biological processes behind the selected endpoints are diverse, involving a different number of intermediary steps (e.g., CYP1A1 induction vs liver toxicity). Also the biological basis and function of the *B* gene is unknown. It may be related to the AHR signaling pathway or an enzymatic difference as well. Therefore, it is a challenge to use dose-response analysis to study the sensitivity differences of various endpoints among rat lines A, B, and C.

Efficacy

We previously demonstrated in a similarly designed study with TCDD-resistant H/W and TCDD-sensitive L-E rats that the short-term effects of TCDD exposure could be divided into two different categories, type I and type II (Simanainen et al., 2002). If the efficacy for an endpoint is similar in resistant and sensitive (wild-type) rats, i.e., efficacy ratio > 0.5 , the endpoint under consideration should be classified as type I. If the efficacy is clearly different, i.e., efficacy ratio < 0.5 , the endpoint should be classified as type II.

The present results with line A, B, and C rats confirm the suitability of efficacy as the classification criterion for short-term effects and reveal that the *Ahr*^{hw} is the most important factor decreasing the TCDD efficacy. When the cutoff value of 0.5 was used for line A:line C efficacy ratio (analogous to H/W:L-E efficacy ratio), thymus weight, EROD activity, and dental defects were classified as type I endpoints. Body weight change, serum bilirubin and FFA levels, and serum ASAT activity were categorized as type II endpoints, as the efficacy was greatly decreased in line A rats. The result is exactly the same as in our previous study with H/W and L-E rats (Simanainen et al., 2002). This indicates that the mechanistic difference between type I and II effects is linked to the *Ahr*^{hw} allele and the altered AHR transactivation domain.

Contrary to the *Ahr*^{hw} allele, the *B*^{hw} allele seems to have only a minor influence on TCDD efficacy, and the endpoint classification in line B is different from that in line A. The line B:line C efficacy ratio was less than or close to 0.5 only for ASAT activity and serum bilirubin. Thus the *B*^{hw} allele may influence TCDD efficacy for serum ASAT and bilirubin levels, but not the other type II endpoints.

Efficacy of a ligand is a measure of the maximal attainable response (E_{\max}) and is influenced by both ligand- and tissue-specific properties. Maximal response depends on the efficiency of receptor coupling, i.e., on the cascade of events, which, from the binding of the ligand to the receptor,

leads to the observed biological effect. Coupling between the receptor and the response are tissue- and endpoint-dependent. The amount and distribution of AHR in line A, B, and C rats have not been studied, but studies in L-E and H/W rats indicated that L-E rats have clearly higher total hepatic levels of AHR than H/W rats (Franc et al., 2001; Pohjanvirta et al., 1999). This difference may contribute to the increased efficacy of TCDD for short-term endpoints. However, no difference between the strains was found in TCDD affinity to cytosolic AHR, in abilities of their AHRs to be transformed into the DNA-binding form by TCDD, or in the specific binding of the activated AHR to DNA (Pohjanvirta et al., 1999). Also the up-regulation of the AHR by TCDD was similar in H/W and L-E rats (Franc et al., 2001).

The deletion mutation of *Ahr*^{hw} is located within the transactivation domain leaving the domains responsible for ligand and DNA binding as well as heterodimerization intact (Pohjanvirta et al., 1998). Therefore, the activation of transcription seems to be the critical step where the wild-type and H/W-type receptors act differently. In vitro and in vivo studies have shown that TCDD-induced binding of AHR-ARNT to the enhancer does not require the C-terminal end of AHR (Ko et al., 1996), but is necessary for formation of a functional AHR conformation that increases promoter accessibility and facilitates promoter occupancy by different transcription factors (Ko et al., 1997; Kronenberg et al., 2000). The promoter sequences and the transcription factor machinery are diverse, involving separate genes. Our results are consistent with the assumption that the mutation in *Ahr*^{hw} selectively affects the formation of functional AHR conformation and communication between enhancer and promoter, and consequently, the expression of different genes.

Potency

This study showed that both H/W-type resistance alleles decreased TCDD potency at least for some endpoints. The different efficacies in type II endpoints among the rat lines and the overlapping ED50 confidence intervals complicate the potency comparisons. However, it is apparent that the differences among the lines were far from those seen in acute lethality.

In this study we measured TCDD effects 8 days postexposure. This was a compromise in aim to observe many different effects. Liver EROD peaks earlier and is already affected by liver toxicity at higher dose-levels on Day 8. However, Håkansson et al. (1994) demonstrated that in Sprague–Dawley rats receiving TCDD up to 67% of their LD50 the EROD induction was maximal from Day 1 to Day 28 after treatment and in C57B1/6 and DBA/2 mice receiving TCDD up to 76% of their LD50 the maximal EROD induction occurred 7 days postexposure. Therefore, in the present study at the doses that were not liver toxic the induction was expected to be maximal. It is therefore likely that the selected time point does not have a major influence

on conclusion derived from the EROD activity model. This conclusion is also supported by the fact that the CYP1A1 response was similar in H/W and L-E rats 3 days after TCDD exposure (Pohjanvirta et al., 1988).

Tooth defects clearly occur at late time points, and the effects are not visible much before Day 8 postexposure. In a recent study we examined H/W and L-E rats after 20-week exposure to TCDD and saw similar responses in the impaired formation of the incisor tooth in both strains (Kiukkonen et al., 2002). Thus, the main result is similar at both time points, implying that these tooth defects are type I effects. Effects on serum bilirubin and ASAT levels are clearly more pronounced at late time points, and 8 days postexposure was considered to be a representative time point for short-term liver effects. Interestingly, Viluksela et al. (2000) showed that in TCDD-resistant H/W and -sensitive L-E rats there is little difference in efficacy but about a 100-fold difference in potency for ASAT activity after 20-week exposure to TCDD. Therefore it seems that the toxicity may accumulate in time also in type II endpoints, disguising the original efficacy difference. This phenomenon is also seen with lethality: although H/W rats are extremely resistant to acute lethality, there is remarkable subchronic mortality after a large dose of TCDD (Viluksela et al., 2000). These data imply that the effect on efficacy is primary, and toxicity accumulation and/or tolerance occurring during several weeks modifies the effect leading to secondary differences in potency.

Line B rats showed dose-responses that do not clearly fall in either type I or II responses. It seems that this classification indicates a difference in the mechanism that is specifically affected by the *Ahr* genotype. Whether gene *B* genotype affects specifically efficacy, potency, or both is not clear. However, the responses differ from line C responses, indicating that gene *B*, in addition to *Ahr*, is somehow involved in these mechanistic pathways.

The identity of gene *B* and the site and mechanism of action are currently under thorough investigation in our laboratory. Tuomisto et al. (1999) showed that the rats heterozygous for both resistance alleles (*Ahr*^{hw/wt} *B*^{hw/wt}) were more resistant than rats heterozygous for either *Ahr*^{hw} (*Ahr*^{hw/wt} *B*^{wt/wt}) or *B*^{hw} (*Ahr*^{wt/wt} *B*^{hw/wt}). This implies that the effects of *Ahr*^{hw} and *B*^{hw} seem to be interactive. Therefore, we hypothesize that *B*^{hw} could be a specific protein participating AHR signaling pathway. A closely related AHR signaling protein is the AHR dimerization partner, AHR nuclear translocator (ARNT). There seem to be several different products of alternative splicing of ARNT mRNA, but no major differences have so far been found in relative expression levels of the variants between H/W and L-E rats or among the A, B, and C lines (Korkalainen et al., 2002; Korkalainen et al., unpublished data). Beischlag et al. (2002) demonstrated recently the importance of NcoA/SRC-1/p160 family of transcriptional activators in TCDD-dependent gene regulation. These observations have led us

to consider these coactivators as well as the AHR repressor as possible candidates for gene *B*.

In conclusion, the results support the concept of at least two different AHR-mediated signaling pathways in short-term dioxin effects, leading to dioxin type I and type II endpoints. Efficacy ratio of 0.5 between *Ahr*^{hw/hw} and *Ahr*^{wt/wt} genotype rats can be used as the classification criterion. The *Ahr*^{hw} allele is the most important factor decreasing the efficacy. For most endpoints the *B*^{hw} allele had only a minor role on efficacy. Both H/W-type resistance alleles also decreased the potency of TCDD. However, the potency differences in short-term toxicity seem not to explain, at least alone, the differences seen in acute lethality among the rat lines.

Acknowledgments

We are grateful for Arja Tamminen and Minna Voutilainen for excellent technical assistance. We thank Dr. Satu Sankari for supervising the clinical chemistry analysis. This study was financially supported by the Academy of Finland, Research Programme on Environmental Health (Contract 42551); Academy of Finland, Research Council for Health (Grant 43984), and European Commission (Contract QLK4-CT-1999-01446); Finnish Cultural Foundation of Northern Savo.

References

- Alaluusua, S., Lukinmaa, P.-L., Pohjanvirta, R., Unkila, M., Tuomisto, J., 1993. Exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin leads to defective dentin formation and pulpal perforation in rat incisor tooth. *Toxicology* 81, 1–13.
- Beischlag, T.V., Wang, S., Rose, D.W., Torchia, J., Reisz-Porszasz, S., Muhammad, K., Nelson, W.E., Probst, M.R., Rosenfeld, M.G., Hankinson, O., 2002. Recruitment of the NcoA/SRC-1/p160 family of transcriptional coactivators by the aryl hydrocarbon receptor/aryl hydrocarbon receptor nuclear translocator complex. *Mol. Cell. Biol.* 22, 4319–4333.
- Birnbaum, L.S., McDonald, M.M., Blair, P.C., Clark, A.M., Harris, M.W., 1990. Differential toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in C57BL/6 mice congenic at the Ah locus. *Fundam. Appl. Toxicol.* 15, 186–200.
- Bradford, M.M., 1976. A rapid and sensitive method for quantitation of microgram quantities of protein using the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254.
- Chapman, D.E., Schiller, C.M., 1985. Dose-related effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in C57BL/6J and DBA/2J mice. *Toxicol. Appl. Pharmacol.* 78, 147–157.
- DeVito, M.J., Diliberto, J.J., Ross, D.G., Menache, M.G., Birnbaum, L.S., 1997. Dose-response relationships for polyhalogenated dioxins and dibenzofurans following subchronic treatment in mice. I. CYP1A1 and CYP1A2 enzyme activity in liver, lung and skin. *Toxicol. Appl. Pharmacol.* 147, 267–280.
- Ema, M., Ohe, N., Suzuki, M., Mimura, J., Sogawa, K., Ikawa, S., Fujii-kuriyama, Y., 1994. Dioxin binding activities of polymorphic forms of mouse and human arylhydrocarbon receptors. *J. Biol. Chem.* 269, 27337–27343.
- FELASA, 1996. Recommendations for the health monitoring of mouse, rat, hamster, gerbil, and guinea pig and rabbit in experimental units. *Lab. Anim.* 30, 193–208.
- Fernandez-Salguero, P.M., Hilbert, D.M., Rudikoff, S., Ward, J.M., Gonzales, F.J., 1996. Aryl-hydrocarbon receptor-deficient mice are resistant to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced toxicity. *Toxicol. Appl. Pharmacol.* 140, 173–179.
- Franc, M.-A., Pohjanvirta, R., Tuomisto, J., Okey, A., 2001. In vivo up-regulation of aryl hydrocarbon receptor expression by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in dioxin-resistant rat model. *Biochem. Pharmacol.* 62, 1565–1578.
- Håkansson, H., Johansson, L., Manzoor, E., Ahlberg, U.G., 1994. Effect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on the hepatic 7-ethoxyresorufin *O*-deethylase activity in four rodent species. *Eur. J. Pharmacol.* 270, 279–284.
- Holford, N.G., Sheiner, L.B., 1981. Pharmacokinetic and pharmacodynamic modeling in vivo. *CRC Crit. Rev. Bioeng.* 5, 273–322.
- Kennedy, S.W., Jones, S.P., 1994. Simultaneous measurement of cytochrome P450A1 catalytic activity and total protein concentration with a fluorescence plate reader. *Anal. Biochem.* 222, 217–223.
- Kiukkonen, A., Viluksela, M., Sahlberg, C., Alaluusua, S., Tuomisto, J.T., Tuomisto, J., Lukinmaa, P.L., 2002. Response of the incisor tooth to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in a dioxin-resistant and a dioxin-sensitive rat. *Toxicol. Sci.* 69, 482–489.
- Ko, H.P., Okino, S.T., Ma, Q., Whitlock Jr., J.P., 1996. Dioxin-induced CYP1A1 transcription in vivo: Ah receptor mediates transactivation, enhancer-promoter communication, and changes in chromatin structure. *Mol. Cell. Biol.* 16, 430–436.
- Ko, H.P., Okino, S.T., Ma, Q., Whitlock Jr., J.P., 1997. Transactivation domains facilitate promoter occupancy for the dioxin-inducible CYP1A1 gene in vivo. *Mol. Cell. Biol.* 17, 3497–3507.
- Korkalainen, M., Tuomisto, J., Pohjanvirta, R., 2002. Striking variability in the structures of ARNT and ARNT2 in the rat. *Toxicol. Sci.* 66, 257.
- Kronenberg, S., Esser, C., Carlberg, C., 2000. An aryl hydrocarbon receptor conformation acts as the functional core of nuclear dioxin signaling. *Nucleic Acid Res.* 28, 2286–2291.
- Okey, A.B., Vella, L.M., Harper, P.A., 1989. Detection and characterization of a low affinity form of cytosolic Ah receptor in livers of mice nonresponsive to induction of cytochrome P₁-450 by 3-methylcholanthrene. *Mol. Pharmacol.* 35, 823–830.
- Pohjanvirta, R., Juvonen, R., Kärenlampi, S., Raunio, H., Tuomisto, J., 1988. Hepatic Ah-receptor levels and the effect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on hepatic microsomal monooxygenase activities in a TCDD-susceptible and -resistant rat strain. *Toxicol. Appl. Pharmacol.* 92, 131–140.
- Pohjanvirta, R., Viluksela, M., Tuomisto, J.T., Unkila, M., Karasinska, J., Holowenko, M., Franc, M.-A., Giannone, J.G., Harper, P.A., Tuomisto, J., Okey, A.B., 1999. Physiocochemical differences in the Ah receptors of the most TCDD-susceptible and the most TCDD-resistant rat strains. *Toxicol. Appl. Pharmacol.* 155, 82–95.
- Pohjanvirta, R., Wong, J.M.Y., Li, W., Harper, P.A., Tuomisto, J., Okey, A.B., 1998. Point mutation in intron sequence causes altered carboxyl-terminal structure in the aryl hydrocarbon receptor of the most 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-resistant rat strain. *Mol. Pharmacol.* 54, 86–93.
- Poland, A., Glover, E., 1975. Genetic expression of aryl hydrocarbon hydroxylase by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin: evidence for a receptor mutation in genetically non-responsive mice. *Mol. Pharmacol.* 11, 389–398.
- Poland, A., Glover, E., Kende, A.S., 1976. Stereospecific, high affinity binding of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin by hepatic cytosol. Evidence that the binding species is receptor for induction of aryl hydrocarbon hydroxylase. *J. Biol. Chem.* 251, 4936–4946.

- Poland, A., Palen, D., Glover, E., 1994. Analysis of the four alleles of the murine arylhydrocarbon receptor. *Mol. Pharmacol.* 46, 915–921.
- Shimizu, S., Tani, Y., Yamada, H., Tabata, M., Murachi, T., 1980. Enzymatic determination of serum free fatty acids: a colorimetric method. *Anal. Biochem.* 107, 193–198.
- Simanainen, U., Tuomisto, J.T., Tuomisto, J., Viluksela, M., 2002. Structure-activity relationships and dose-responses of polychlorinated dibenzo-*p*-dioxins for short-term effects in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-resistant and -sensitive rat strains. *Toxicol. Appl. Pharmacol.* 181, 38–47.
- The Committee on Enzymes of the Scandinavian Society of Clinical Chemistry and Clinical Physiology, 1974. Recommended methods for the determination of four enzymes in blood. *Scand. J. Clin. Lab. Invest.* 33, 291–306.
- Tuomisto, J.T., Viluksela, M., Pohjanvirta, R., Tuomisto, J., 1999. The AH receptor and a novel gene determine acute toxic responses to TCDD: segregation of the resistant alleles to different rat lines. *Toxicol. Appl. Pharmacol.* 153, 71–81.
- Viluksela, M., Bager, Y., Tuomisto, J.T., Scheu, G., Unkila, M., Pohjanvirta, R., Flodström, S., Kosma, V.-M., Mäki-Paakkanen, J., Vartiainen, T., Klimm, C., Schramm, K.-W., Wärngård, L., Tuomisto, J., 2000. Liver tumor-promoting activity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in TCDD-sensitive and TCDD-resistant rat strains. *Cancer Res.* 60, 6911–6920.
- Walters, M., Gerarde, H., 1970. An ultramicromethod for the determination of conjugated and total bilirubin in serum or plasma. *Microchem. J.* 15, 231–243.